

Proteolytically-cleaved Fragments of Cell Surface Proteins Stimulate a Cytotoxic Immune Response Against Tumor-activated Endothelial Cells *In vitro*

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Abstract

Endothelial antigens that stimulate immune-mediated damage of tumor vessels represent possible targets for the development of antiangiogenic vaccines aimed at preventing the progression of solid tumors. Since antigens expressed on the cell surface are accessible targets for both humoral and cell-mediated immune responses, the ability to isolate extracellular protein fragments from endothelial cells by proteolytic digest is a proposed strategy for the creation of antiangiogenic vaccines. Human microvascular endothelial cells (HMEC) were isolated from an abdominal subcutaneous adipose tissue biopsy. Both non-activated endothelial cells (nHMEC) and tumor-activated endothelial cells (aHMEC) were obtained. HMEC lysate and cleaved fragments of cell surface proteins (FCSP) of HMEC had total protein concentrations of 135 µg/mL and 2 µg/mL, respectively. Despite this difference in concentration, FCSP were able to stimulate immune cells in cytotoxicity assays better than the HMEC lysate. Moreover, FCSP obtained from tumor-activated endothelial cells were able to stimulate an immune response toward tumor-activated endothelial cells. Based on these results, FCSP of endothelial cells appear to provide a comprehensive set of surface antigens that are able to induce targeted, immune-mediated cytotoxic effects against tumor endothelial cells. These findings represent a successful strategy to produce safe and pure antigens for the production of antiangiogenic vaccines.

Keywords: Antiangiogenic cancer vaccine; Tumor-associated antigens; Endothelial cells

Abbreviations: DC: Dendritic Cells; PBMC: Peripheral Blood Mononuclear Cells; CTL: Cytotoxic T Lymphocytes; HMEC: Human Microvascular Endothelial Cells; nHMEC: Non-Activated Endothelial Cells; aHMEC: Tumor-activated Endothelial Cells; FCSP: Fragments of Cell Surface Proteins

Introduction

The secretion of angiogenic stimulators by malignant cells activates endothelial cells to proliferate and form new blood vessels, and this additional vascular capacity is essential for tumor growth and metastasis (Carmeliet and Jain, 2000; Ellis and Fidler, 1996; Folkman, 1971; Folkman, 1990; Liotta et al., 1991; Pluda, 1997; Pralhad et al., 2003). The expression of antigens associated with tumor-stimulated angiogenesis represents distinguishing traits of angiogenic endothelial cells versus endothelium of the normal vasculature (Bhati et al., 2008; Khodarev et al., 2003; St Croix et al., 2000). Various approaches have been used to target angiogenic vasculature, including monoclonal antibodies and synthetic molecules, which have been shown to inhibit tumor growth in animal models and have been administered in clinical trials (Arap et al., 1998; Folkman, 2007; Liao et al., 2002; Scappaticci, 2002; Schraa et al., 2002; Shaheen et al., 2001). However, these antiangiogenesis therapies can be expensive if a high dose and extended administration period is needed to compensate for a short half-life of these therapeutics. In addition, antigens highly specific for angiogenic endothelium remain elusive. An active immunization with endothelial cells overcomes these limitations. This vaccination approach offers the advantage that targeting of autologous antigens and previously uncharacterized antigens can be achieved without needing to isolate the relevant targets. Previously this approach has been shown to inhibit the growth of experimental tumors in mouse models (Chen et al., 2006; Corsini

et al., 2004; Okaji et al., 2004; Okaji et al., 2006; Scappaticci and Nolan, 2003; Wei et al., 2000). However, there are additional considerations to this approach. For example, while whole cells provide surface antigens that induce a desired immune response, they also contain a high abundance of housekeeping proteins, carbohydrates, nucleic acids, and other intracellular contents that are commonly expressed by all mammalian cells. These ubiquitous molecules would be undesirable targets for an immune response, and represent a possible reason for the failure of cell-based vaccines (Cohen et al., 2009; Copier and Dalgleish, 2010). Another potential limitation of cell-based vaccines includes the difficulty associated with purifying cells from intracellular contaminants such as cell parasites, viruses, toxins, and prions. Successful purification would be essential for the administration of anti-cancer vaccines (Emens, 2006; Levine and Szein, 2004).

One potential approach to the production of antiangiogenic vaccines is to isolate essential targets localized to the endothelial cell surface. Since the immune response is usually based on the recognition of extracellular cell surface molecules, proteins, and glycoproteins, proteolytic cleavage represents the ability to isolate these protein groups from the cell surface. While the antigen profile obtained by proteolytic cleavage would contain a comprehensive

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set of native antigens, the immune system could be directed to recognize a subset of these antigens that do not represent ubiquitous molecules presented in all normal cells. Therefore, this study investigates the ability of a trypsin treatment to proteolytically cleave the cell surface antigens of human microvascular endothelial cells (HMEC), and the use of these cleaved protein fragments to stimulate immune-mediated cytotoxic responses against tumor-activated HMEC *in vitro*.

Materials and Methods

Primary culture of HMEC

An abdominal subcutaneous adipose tissue biopsy was obtained from a male patient undergoing an open-abdominal surgical procedure at the National Medico-Surgical Center (Moscow, Russia). The protocol was approved by the Research Ethics Committee and the patient provided his written informed consent. The biopsy specimen was transported to the laboratory in Ringer solution (transport time 45 min), and after removal of the visible fibrous tissue, the fat was finely minced and incubated for 45h at 37°C in digest solution (0.5 mg/ml collagenase IA (Sigma-Aldrich, USA) prepared with Hank's Balanced Salt Solution (HBSS)) at a ratio of 4:1 (v/v) (Hutley et al., 2001). The digested material was then intensely shaken for 2 min then centrifuged (300 × *g* for 10 min) to separate adipocytes and free oil from the stromovascular components. The stromovascular pellet was resuspended in HBSS and washed 3 x by centrifugation (600 × *g* for 5 min). The resulting pellet was incubated in 0.25% trypsin (activity 300 U/mg, PanEco, Russia) containing 1 mM EDTA for 15 min at RT, followed by 3 washes with PBS containing 0.1% bovine serum albumin (BSA) by centrifugation (600 × *g* for 5 min). Endothelial cells were isolated using CD31 Dynabeads (Invitrogen, USA) according to the manufacturer's protocol, and seeded onto 1% gelatin-coated 6-well tissue culture plates in selective endothelial cell growth medium (DMEM with D-valine (PanEco, Russia), 20% fetal bovine serum (PAA Laboratories, USA), 100 U penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 12 U/ml heparine, 50 µg/ml endothelial cell growth supplement from bovine pituitary (Sigma-Aldrich)) at 37°C in 7.5% CO₂. Culture media was changed every 2-3 days and after the first passage, cells were grown to 75% confluence and used in further experiments as non-activated endothelial cells (nHMEC). To obtain tumor-activated endothelial cells (aHMEC), cell cultures were incubated for 2 days in medium collected from MCF-7 human breast adenocarcinoma cells (ATCC, Manassas, VA) as described by Folkman et al. (1979). Briefly, growth medium was aspirated and replaced with the medium to be conditioned. After 48h, the medium was collected, centrifuged for 10 min at 600 × *g*, filter sterilized (0.2 µm), and added to cultures.

Preparation of FCSP from HMEC

HMEC grown to 75% confluence were washed 5 × with HBSS before being treated with 0.2 µg/mL trypsin (15000 U/mg, Promega, Madison, WI) in HBSS. One mL of trypsin solution was added to each 25 cm² flask, incubated for 20 min at 37°C in saturated humidity, then collected again and centrifuged (600 × *g* for 5 min). The resulting supernatant contained fragments of cell surface proteins (FCSP) from HMEC, and total protein concentration for this FCSP preparation was determined using a BCA™ Protein Assay Kit (Thermo Scientific, USA).

Cell growth medium containing 20% FCS was added to the treated HMEC and cell viability was determined directly in flask

using the trypan blue exclusion method (Hudson and Hay, 1980). Viability of cells that became detached during trypsin digestion was also taken into account. The number of viable cells present was calculated by subtracting an average number of cells stained with trypan blue from the average number of cells observed in 5 separate fields.

Preparation of HMEC lysate

HMEC grown to 75% confluence in 25 cm² flasks were washed 5 x with HBSS then detached with a cell scraper in 1 mL HBSS. Collected cells were freeze-thawed three times (Nestle et al., 1998) then centrifuged for 5 min at 600 × *g*. Protein concentrations were determined using a BCA™ Protein Assay Kit and supernatants were used to load DC.

Preparation of cell lysate- and FCSP-loaded DC

Monocyte-derived DC were generated as described previously (Romani et al., 1994). Briefly, fresh peripheral blood mononuclear cells (PBMC) from healthy donors were isolated using Ficoll-Hypaque (PanEco, Russia) gradient centrifugation and were then allowed to adhere to culture flasks for 1h. Non-adherent cells were collected and centrifuged, and cell pellets were mixed with autologous serum containing 10% DMSO and stored in liquid nitrogen. Cryopreserved, non-adherent PBMC were later used as a source of effector cells (cytotoxic T lymphocytes, CTL) for cytotoxicity assays. The adherent cell fraction was cultured in RPMI-1640 (PanEco) supplemented with 10% FBS (PAA Laboratories) in the presence of 1000 U/mL granulocyte macrophage colony-stimulating factor (Sigma-Aldrich) and 1000 U/mL interleukin-4 (Sigma-Aldrich). After 6 d in culture, either HMEC lysate (250 µg/mL, 1 mL) or FCSP (3 µg/mL, 1 mL) were combined with an equal volume (1 mL) of immature DC. Antigen loading was allowed to occur for 3h, then DC were matured with 1000 U/mL tumor necrosis factor- α (Sigma-Aldrich) for 48h. Matured, lysate- or FCSP-loaded DC were then used to stimulate CTL. For successive cycles of CTL stimulation, aliquots of matured and loaded DC were cryopreserved and thawed as needed. The freezing method used has been previously described (John et al., 2005).

Stimulation of CTL

Lysate- or FCSP-loaded DC (4.5×10^4) were washed with HBSS and cultured in 6-well plates. DC were combined with 9×10^5 autologous non-adherent PBMC (1:20) in 4 mL of RPMI-1640 medium supplemented with 30 U/mL clinical grade human interleukin-2 (Ronkoleukin, Russia) and 10% FBS, and this medium was replaced every third day. The second portion of antigen-loaded DC was added to PBMC 7 d after stimulation. After another 5 d, non-adherent PBMC containing stimulated CTL were washed by centrifugation and used in cytotoxicity assays as effector cells.

Cytotoxicity assays

HMEC (2×10^4 cells/well) were seeded into 48-well plates, which yielded 3.7×10^4 cells/well after 48h. Effector CTL were then added to HMEC at an effector: target ratio of 8:1. On the 3rd day, target HMEC were washed to remove CTL and were photographed using an inverted phase contrast microscope. In duplicate wells, attached HMEC were trypsinized and viability was detected using trypan blue exclusion. Cell counts were averaged over three measurements and the number of HMEC in the absence of effector CTL, as well as with CTL that had been stimulated with unloaded DC, were used as controls. Statistically significant differences were determined using Student's t-test.

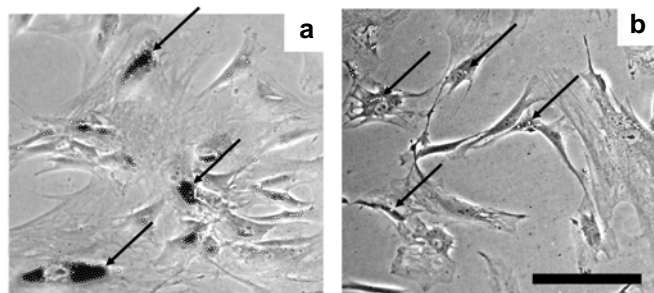


Figure 1: Primary culture of human microvascular endothelial cells (HMEC). A representative colony of microvascular endothelial cells associated with anti-CD31 beads 24h after isolation (a), and after the first passage (b). There were no cells not associated with CD31 beads that would represent contaminating cells. Images were obtained using an inverted phase contrast microscope (scale bar: 50 μ m). Arrows show beads attached to cells.

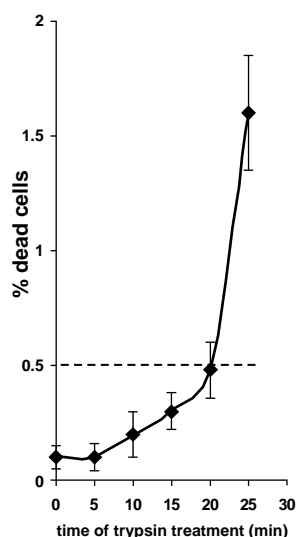


Figure 2: HMEC viability following treatment with trypsin. *In situ* trypan blue staining of endothelial cell cultures at various timepoints during treatment with 0.2 μ g/mL trypsin (activity 1500 U/mg). The percentage of dead cells were calculated as the average number of stained cells \pm SD per field with the result from each of the five fields being averaged. The dotted line shows percent of dead cells corresponding to time which has been selected for obtaining of FCSP.

Results

Primary culture of HMEC

To isolate micro vascular endothelial cells from a biopsy of abdominal fat obtained, anti-CD31 beads were used as described in the Materials and methods. Figure 1a shows a colony of endothelial cells isolated after 24h with numerous beads still attached to the isolated cells. After 7 d, the endothelial cells isolated formed a confluent monolayer, with the number of beads bound per cell decreasing with each replication cycle. As shown in Figure 1b, beads were still attached to endothelial cells when the first passaging following isolation was performed, yet no evidence of overgrowth by contaminating cells was detected. Thus, a primary culture of HMEC was established.

Preparation of FCSP

To select conditions optimal for the cleavage of extracellular proteins from HMEC, *in situ* trypan blue staining of HMEC was used

to assay the cell death rate following treatment with a $2 \times 10^{-5}\%$ solution of highly purified (activity 15000 U/mg) trypsin. The cell death rate did not exceed 0.5% after incubation of the HMEC with trypsin for 20 min, therefore, these conditions were selected for the preparation FCSP. The total protein concentration for preparations of FCSP vs. HMEC lysates was 2 μ g/mL vs. 135 μ g/mL, respectively.

Cytotoxicity assays

The immunologic properties of the FCSP were also evaluated by loading DC with the digested material and stimulating normal human cytotoxic T lymphocytes (CTL). In these cytotoxicity assays, unloaded DC were used as controls and lysate-loaded DC were used as reference DC. CTL stimulated with FCSP-loaded DC were observed to kill target HMEC as effectively as CTL stimulated with lysate-loaded DC. Similar observations were made for non-activated and activated HMEC used as target cells (Figure 3, c-f & c'-f', respectively).

On day 3, surviving endothelial cells were detected using trypan blue exclusion (Figure 4) and a slight increase in cytotoxic activity was observed when CTL stimulated with unloaded DC were incubated with target HMEC (Figure 4 b, b'). CTL stimulated with DC loaded with FCSP were more effective than CTL stimulated with DC loaded with lysate in killing target HMEC at each assay point (Figure 4, c vs. e, d vs. e', c' vs. e', and d' vs. f'; $p < 0.05$ for d' vs. f'). Furthermore, DC loaded with FCSP prepared from activated endothelial cells resulted in more effective killing activated than non-activated target endothelial cells (Figure 4 e' vs. f', $p < 0.01$). Similar results were obtained when DC loaded with FCSP prepared from non-activated endothelial cells toward activated or non-activated targeted endothelial cells, and killing was more effective in the latter case (Figure 4 e vs. f, $p < 0.05$).

Discussion

Many studies have shown that cancer cells can provide a comprehensive set of native antigens suitable for inducing an immune response (Chiang et al., 2010; de Gruijl et al., 2008; Thompson and Dessureault, 2007), therefore, a similar approach has been proposed for the use of endothelial cells to develop antiangiogenic, anti-cancer vaccines. However, numerous shortcomings have been associated with the use of whole endothelial cells. For example, while endothelial cells provide the extracellular macromolecules, which are accessible to antibodies and cytotoxic cells of the immune system, they also include intracellular substances which are ubiquitous in all mammalian cells, including normal endothelial cells (Cohen et al., 2009). Another crucial shortcoming is the possible presence of cell parasites, viruses, toxins, or prions in cells, which would make the administration of any whole cell preparations dangerous. Therefore, an approach which allows a subset of extracellular antigens to be isolated and used to develop vaccines is needed.

In this study, cell surface antigens were obtained from primary culture of HMEC. By carefully manipulating the cells and using a highly purified protease, antigenic targets present on the cell surface, referred to as FCSP, were isolated. The decision to use HMEC in these assays was based on previous characterizations of endothelial cells involved in tumor angiogenesis which predominantly identified endothelial cells of micro vascular origin. In addition, micro vascular endothelial cells exhibit a number of functional differences compared to large vessel-derived endothelial

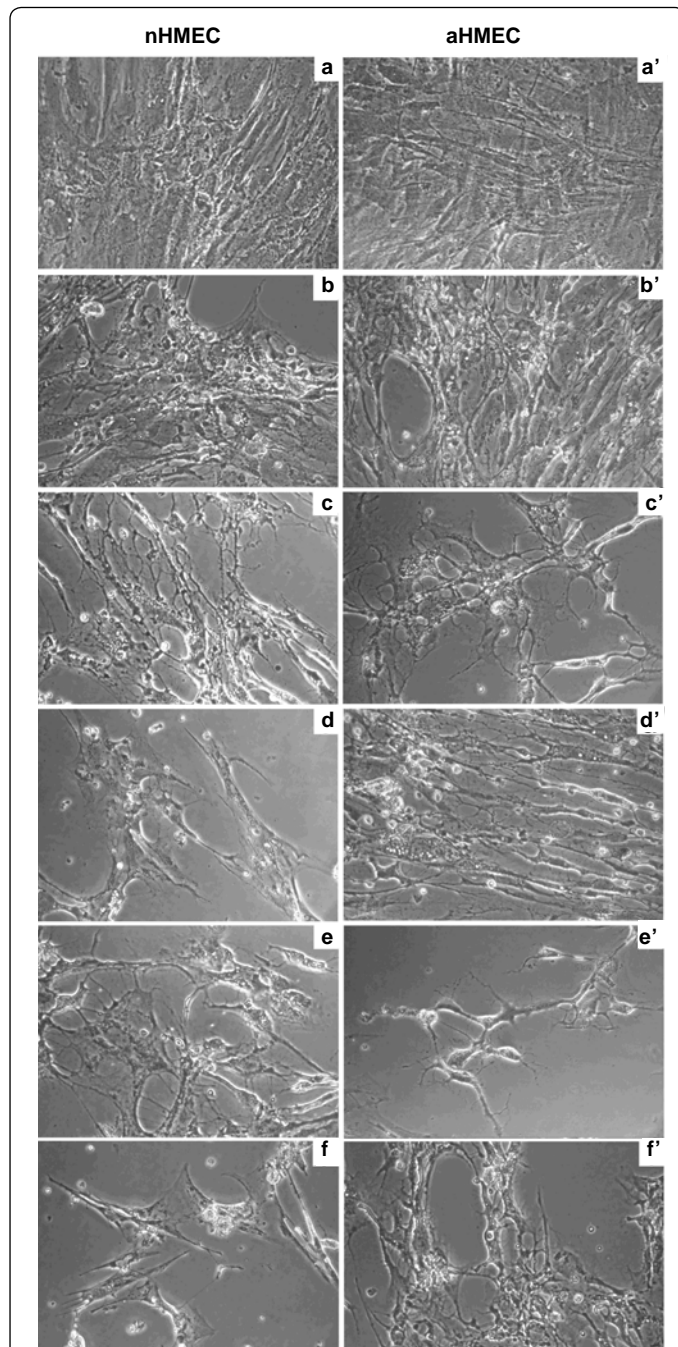


Figure 3: CTL-mediated lysis of HMEC in cytotoxicity assays. Non-activated HMEC (nHMEC) and tumor-conditioned medium activated HMEC (aHMEC) (3.7×10^4 cells/well) were incubated with effector CTL at a ratio of 1:8 and were imaged using an inverted phase contrast microscope. Untreated nHMEC and aHMEC (a, a') are compared with nHMEC and aHMEC incubated with: CTL stimulated with control (unloaded with antigens) DC (b, b'), CTL stimulated with DC loaded with aHMEC lysate (c, c'), CTL stimulated with DC loaded with nHMEC lysate (d, d'), CTL stimulated with DC loaded with FCSP prepared from aHMEC (e, e'), and CTL stimulated with DC loaded with FCSP prepared from nHMEC (f, f'). For panels a-f, nHMEC are the target cells, and in panels a'-f', aHMEC are the target cells.

cells (Kumar et al., 1987; Lang et al., 2003), including their response to stimulators (Hewett, 2001; Shreeniwas et al., 1991) and their regulation of extracellular protein expression (Lee et al., 1992; Swerlick et al., 1992a; Swerlick et al., 1992b). Although the isolation

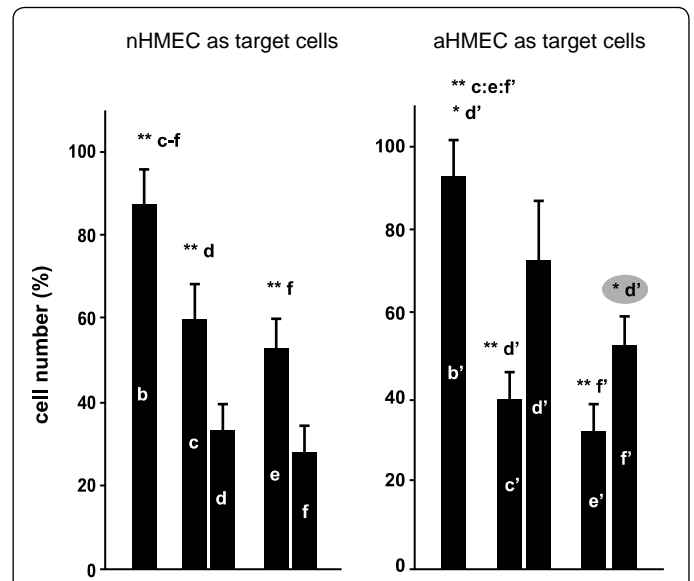


Figure 4: Cytotoxicity of effector CTL against HMEC. Target HMEC cells (3.7×10^4 cells/well) were incubated with effector CTL at a ratio of 1:8. After 3 days, floating CTL were removed, target cells were carefully washed with HBSS, and attached HMEC were trypsinized and cell viability determined. Bars correspond to images in Figure 4 according to literal notation. The number of calculated HMEC present in control cultures was set as 100%. ** - $p < 0.01$; * - $p < 0.05$, additional marks indicate bars paired to calculate p . Data represent the mean value of 3 identical measurements \pm SD.

and culture of micro vascular endothelial cells is difficult due to the potential for contamination from other cell types, including mesothelial cells (Hewett et al., 1993; Hull et al., 1996; Potzsch et al., 1990), the use of magnetic beads coated with anti-CD31 antibodies was shown in this study to address this problem since HMEC were the predominant population expressing CD31 (Figure 1), which is consistent with the results of other studies (Hewett and Murray, 1994; Hull et al., 1996).

The use of proteases to isolate a subset of cell surface antigens for anti angiogenesis vaccines is only useful if the treatment with proteases is associated with a low rate of cell death. This is necessary to avoid the contamination of the isolated extracellular antigens with the intracellular contents released from dead cells. In this study, a cell death rate of less than 0.5% was achieved following treatment with trypsin (Figure 2). Moreover, the protein content of the FCSP preparations was found to be $2 \mu\text{g/mL}$, which was significantly lower than the protein content of HMEC lysates ($135 \mu\text{g/mL}$). This difference in the concentration of antigens detected is due to the presence of both extracellular and intracellular proteins in the HMEC lysates, while the FCSP preparations only contain proteins that were cleaved from the cell surface. This is consistent with data reported in a previous study where $\sim 5 \mu\text{g}$ of protein was isolated from the plasma membrane of 1×10^6 mammalian cells (Deblaquiere and Burgess, 1999).

To confirm that the FCSP contained essential antigens for vaccination, the immunogenic properties of both FCSP preparations and cell lysates were compared. Using an *in vitro* model of human antiangiogenic therapies where human CTL were incubated with endothelial cell targets, DC loaded with FCSP were found to stimulate the cytotoxic actions of CTL just as effectively as DC loaded with lysates (Figure 4). Therefore, despite the much lower concentration of total protein associated with the FCSP

preparations, the same ability to induce cytotoxic activity in CTL indicated that the necessary antigens were present in the absence of intracellular proteins. Correspondingly, this proof-of-concept study demonstrated that the treatment of endothelial cells with trypsin can release antigenic targets that are sufficient to induce antiangiogenic, antitumor immune responses *in vitro*.

Endothelial cells in tumors that are stimulated to proliferate in response to tumor-secreted stimulators, undergo changes that make them distinct from endothelial cells of the normal vasculature, particularly in regard to expression of cell surface macromolecules (Ruoslahti and Rajotte, 2000; St Croix et al., 2000). By performing cytotoxicity assays with tumor-activated HMEC as the target cells, the capacity for the treatment of HMEC with trypsin to provide antigens for the stimulation of the immune response specifically for tumor endothelial cells was demonstrated. Similarly, activation of endothelial cells by tumor-conditioned medium has previously been shown to be a widely accepted *in vitro* model of tumor-activated endothelial cells (Folkman and Haudenschild, 1980; Folkman et al., 1979; Li et al., 2003; Papetti and Herman, 2002).

CTL stimulated with DC loaded with FCSP obtained from activated endothelial cells were associated with a greater cytotoxicity than non-activated HMEC. Moreover, CTL stimulated with DC loaded with FCSP vs. lysates obtained from non-activated endothelial cells were more cytotoxic, respectively, toward non-activated HMEC. These results suggest that FCSP of tumor-activated HMEC contain the relevant antigens to stimulate the immune response toward tumor endothelial cells. In particular, it is remarkable that FCSP stimulated the immune response better than lysates, and when aHMEC were used as the target cells, the stimulation by FCSP was statistically significant (see ellipse mark in Figure 4). We hypothesize that this is due to the absence of intracellular substances in the FCSP preparations which would otherwise interfere with target antigens during the loading of DC, thereby leading to a less targeted immune response.

Conclusions

While additional studies are need to identify the optimal choice of adjuvant and administration schedule for vaccine development, the diversity of native endothelial-associated antigens obtained, the low levels of undesirable antigens present, and the strong immune response stimulated, are all factors that demonstrate the potential for FCSP to provide antiangiogenic, anti-cancer vaccines.

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